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(23*S*)-1,23,25-Trihydroxyvitamin D_3: Its Biologic Activity and Role in 1α,25-Dihydroxyvitamin D_3 26,23-Lactone Biosynthesis†

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ABSTRACT: (23*S*)-1,23,25-Trihydroxyvitamin D_3 was isolated from bovine kidney homogenates incubated with 1,25-dihydroxyvitamin D_3 by sequential chromatography through one Sephadex LH-20 column and three high-performance liquid chromatography systems. Ultraviolet absorption spectroscopy and mass spectrometry confirmed the structural assignment. One high-performance liquid chromatography system separated the *R* and *S* epimers of 1,23,25-trihydroxyvitamin D_3 and indicated that the natural product had the *S* configuration. Plasma pharmacokinetic studies in rats showed that (23*S*)-1,23,25-trihydroxy[^{3}H]vitamin D_3 was rapidly cleared from plasma ($t_{1/2}$ = 60 min). 1α,25-Dihydroxy[^{3}H]vitamin D_3 26,23-lactone appeared concurrently with the disappearance of (23*S*)-1,23,25-trihydroxy[^{3}H]vitamin D_3. Experiments with radioinert compounds showed that 1,25-dihydroxyvitamin D_3 and (23*S*)-1,23,25-trihydroxyvitamin D_3 were efficient precursors to 1,25-dihydroxyvitamin D_3 26,23-lactone both in intact and in nephrectomized rats. (25*S*)-1,25,26-Trihydroxyvitamin D_3, however, was ineffective at raising plasma 1,25-dihydroxyvitamin D_3 26,23-lactone concentrations. These results confirm the presence of extrarenal 1,25-dihydroxyvitamin D_3 23(*S*)-hydroxylase(s) and demonstrate that C-23 hydroxylation of 1,25-dihydroxyvitamin D_3 precedes C-26 hydroxylation in the formation of 1,25-dihydroxyvitamin D_3 26,23-lactone. (23*S*)-1,23,25-Trihydroxyvitamin D_3 had no intestinal calcium absorptive or bone calcium resorptive activity when dosed to vitamin D deficient rats at levels up to 500 ng. C-23 oxidation, therefore, appears to be a physiologic pathway of 1,25-$(OH)_2D_3$ metabolism and a major pathway for the deactivation of pharmacologic levels of 1,25-dihydroxyvitamin D_3.

During normal nutrition or hypervitaminosis D, the most abundant vitamin D metabolite in the plasma is 25-hydroxyvitamin D (25-OHD).[1] Oxidation of 25-OHD at C-1, C-24, and C-26 results in the formation of 1,25-$(OH)_2D_3$, (24*R*)-24,25-$(OH)_2D_3$, and (25*S*)-25,26-$(OH)_2D_3$. These metabolites circulate in plasma under physiological conditions (Horst et al., 1981a; Napoli et al., 1981; Partridge et al., 1981a). A hormonal form of vitamin D_3, 1,25-$(OH)_2D_3$, is similarly metabolized at C-24 and C-26 to form (24*R*)-1,24,25-$(OH)_3D_3$ and (25*S*)-1,25,26-$(OH)_3D_3$ (Holick et al., 1973; Partridge et al., 1981b; Reinhardt et al., 1981, 1982). Under normal vitamin D nutrition, nephrectomy prevents the formation of 24-hydroxylated vitamin D_3 metabolites and lactone. However, 24-oxidized compounds and lactone reappear in nephrectomized animals given an excess of vitamin D_3 (Horst et al., 1981a; Horst & Littledike, 1980).

The first evidence for vitamin D C-23 oxidative pathways surfaced with the identification of the lactones of 25-OHD_3 and 1,25-$(OH)_2D_3$ (Horst, 1979; Wichmann et al., 1979; Ishizuka et al., 1981). Subsequently, the compounds (23*S*)-23,25-$(OH)_2D_3$ and (23*R*)-23,25-$(OH)_2D_3$ were chemically

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[1] Abbreviations: 25-OHD, 25-hydroxyvitamin D; 1,25-$(OH)_2D_3$, 1,25-dihydroxyvitamin D_3; 24,25-$(OH)_2D_3$, 24,25-dihydroxyvitamin D_3; 25,26-$(OH)_2D_3$, 25,26-dihydroxyvitamin D_3; 1,24,25-$(OH)_3D_3$, 1,24,25-trihydroxyvitamin D_3; 1,25,26-$(OH)_3D_3$, 1,25,26-trihydroxyvitamin D_3; 25-OHD_3, 25-hydroxyvitamin D_3; 23,25-$(OH)_2D_3$, 23,25-dihydroxyvitamin D_3; lactone, 25-hydroxyvitamin D_3 26,23-lactone; 1,23,25-$(OH)_3D_3$, 1,23,25-trihydroxyvitamin D_3; HPLC, high-performance liquid chromatography; GC/MS, gas chromatography/mass spectrometry; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; EI, electron impact.

synthesized (Partridge et al., 1982) and used to demonstrate that the naturally occurring epimer of 23,25-$(OH)_2D_3$ present in vitamin D_3 toxic pig plasma had the 23*S* configuration (Napoli et al., 1982). Originally, some controversy existed as to the lactone precursor. In vitro, 25,26-dihydroxyvitamin D_3 has been shown to be a minor metabolic precursor to lactone (Hollis et al., 1980; Tanaka et al., 1980); however, other experiments conclusively demonstrated that in vivo C-23 oxidation preceded C-26 hydroxylation as the major pathway and that (25*R*)-25,26-$(OH)_2D_3$, but not (25*S*)-25,26-$(OH)_2D_3$, could act as minor precursor to lactone (Napoli & Horst, 1981, 1982). An analogous pathway can be anticipated for the formation of 1α-lactone from 1,25-$(OH)_2D_3$. Tanaka et al. (1980) concluded that 1,25-$(OH)_2D_3$ is not converted into 1α-lactone, an observation contrary to that of Ishizuka et al. (1981). Napoli et al. (1983) have recently shown that, under normal physiologic conditions, ^{3}H-1α-lactone is a minor product of 1,25-$(OH)_2[^3H]D_3$ metabolism but animals receiving pharmacologic doses of 1,25-$(OH)_2D_3$ for several days prior to dosing with 1,25-$(OH)_2[^3H]D_3$ produced large amounts of ^{3}H-1α-lactone. The anticipated intermediate to 1α-lactone biosynthesis, (23*S*)-1,23,25-$(OH)_3D_3$, has been detected in intestinal homogenates incubated with 1,25-$(OH)_2[^3H]D_3$ (Napoli & Horst, 1983a). This paper reports isolation of (23*S*)-1,23,25-$(OH)_3D_3$ from bovine kidney homogenates incubated with 1,25-$(OH)_2D_3$, physical and spectroscopic characterization of (23*S*)-1,23,25-$(OH)_3D_3$, and C-23 oxidation in kidney and in extrarenal tissue as the first step in the formation of 1α-lactone from 1,25-$(OH)_2D_3$.

Materials and Methods

High-performance liquid chromatography (HPLC) was performed with Waters Associates Model ALC/GPC 204 liquid chromatography equipment (Waters Associates, Milford, MA). Vitamin D compounds were detected at 254 nm. HPLC columns were purchased from Du Pont Instruments (Wilmington, DE), unless otherwise noted. Normal phase refers to microparticulate silica gel columns. Reverse phase refers to microparticulate silica gel columns derivatized with octadecylsilane (ODS). HPLC solvents were purchased from Burdick & Jackson Laboratories (Muskegon, MI). Ultraviolet spectra were recorded in ethanol with a Beckman Model 25 recording spectrophotometer. Molar extinction coefficients of 18 200 and 19 400 were used for vitamins D_3 and D_2, respectively.

Mass spectra were obtained at 70 eV from the solids probe of a Finnigan Model 4021 EI/CI GC/MS system coupled with an INCOS 2000 data system. To obtain spectra, the probe was heated from ambient to 320 °C over a 10-min period at an ionizer temperature of 250 °C. Chloride addition, negative ion, chemical ionization (Cl-NCI) mass spectra were obtained with dichlorodifluoromethane as reagent gas.

Compounds. Vitamins D_2 and D_3 were purchased from Sigma Chemical Co. (St. Louis, MO). 23(*S*),25(*R*)-1α-Lactone, (25*S*)-1,25,26-$(OH)_3D_3$, (23*S*)-1,23,25-$(OH)_3D_3$, and 1,25-$(OH)_2D_3$ were generous gifts from Drs. J. J. Partridge and M. R. Uskokovic of Hoffmann-La Roche, Inc. (Nutley, NJ) (Partridge et al., 1976, 1982). 1,25-OH-[26,27-^{3}H]D_3 (90 Ci/mmol) was synthesized by reacting 1α-hydroxy-27-nor-25-ketovitamin D_3 with [^{3}H]methylmagnesium bromide (90 Ci/mmol). Its purity, specific activity, and biological activity were verified by HPLC and by its reactivity in the intestinal and thymus 1,25-$(OH)_2$D receptor protein binding assays (Horst et al., 1981b; Reinhardt et al., 1983). (23*R*)-1,23,25-Trihydroxyvitamin D_3 was prepared as previously described (Napoli & Horst, 1983a).

Production and Isolation of 1,23,25-$(OH)_3[^3H]D_3$ and (23S)-1,23,25-$(OH)_3D_3$ from Bovine Kidney Homogenates. Kidneys were collected from 6- to 8-week-old male Jersey calves fed a stock diet. At 24 h and again 4 h before sacrifice, the animals received intramuscularly 25 μg of 1,25-$(OH)_2D_3$ in ethanol. The kidneys were collected and placed in cold buffered sucrose (0.25 M sucrose, 0.05 M HEPES, pH 7.4). Homogenization and incubation conditions were as described by Engstrom et al. (1984). Briefly, 15 mL of a 20% homogenate of kidney cortex in buffered sucrose was centrifuged at 45000*g* for 10 min. The crude pellet was resuspended in 15 mL of buffered sucrose. A total of 11 incubation flasks were prepared. Each contained 1 mL of the crude pellet suspension, 1 mL of HEPES buffer (0.05 M, pH 7.4), 0.5 mL of 0.02 M $MgCl_2$, 0.5 mL of 0.1 M malate, and 2 mL of 0.25 M sucrose. The flasks were purged with O_2 for 30 s and were allowed to incubate unstoppered for 5 min at 37 °C. After the initial 5-min incubation, 1 mCi of 1,25-$(OH)_2$[26,27-^{3}H]D_3 (5 μg) was added to one flask and 15 μg of 1,25-$(OH)_2D_3$ was added to each of the other 10 flasks. The reactions were quenched after 1 h by the addition of 15 mL of methanol/dichloromethane (2:1). The solvent was evaporated from the organic phase. The residue from the incubations of unlabeled 1,25-$(OH)_2D_3$ was suspended in 4 mL of water/chloroform/methanol/hexane (0.015:1:1:9) and applied in equal amounts to four separate Sephadex LH-20 columns developed in the same solvent. The first 20 mL of solvent was recovered, which contained the neutral lipids and 1,25-$(OH)_2D_3$. The trihydroxyvitamin D_3 metabolites and 1α-lactone were eluted by the addition of 15 mL of water/chloroform/methanol/hexane (0.015:2:2:8). These eluents were combined, and the solvent was removed under N_2. The partially purified residue was then applied onto a semipreparative, normal-phase HPLC column (0.9 × 25 cm) developed in 2-propanol/hexane (1:9). In this system, 1,25-$(OH)_2D_3$, 1,23,25-$(OH)_3D_3$, 1,24,25-$(OH)_3D_3$, and 1α-lactone elute at 75, 90, 115, and 152 mL, respectively. The 1,23,25-$(OH)_3D_3$ and 1,24,25-$(OH)_3D_3$ were collected together and were applied to a normal-phase analytical column developed in 2-propanol/methylene chloride (7:93). The 1,23,25-$(OH)_3D_3$ and 1,24,25-$(OH)_3D_3$ migrated at 32 and 38 mL, respectively. The 1,23,25-$(OH)_3D_3$ was collected and was applied to a Zorbax-NH_2 column (0.46 × 25 cm) developed in 2-propanol/hexane (14:86). 1,23,25-$(OH)_3[^3H]D_3$ was isolated from the incubations containing the 1,25-$(OH)_2[^3H]D_3$ in exactly the same manner.

In Vivo Experiments: 1α-Lactone Precursors. Experiments to determine 1α-lactone precursors were done with male Sprague-Dawley rats (Holtzman Co., Madison, WI), 250 ± 20 g, dosed orally for 14 days with vitamin D_2 (1.25 mg) in cottonseed oil (0.1 mL). Twenty-four hours after the last dose, each animal received 2 μg of the appropriate vitamin D_3 metabolite intraperitoneally in propylene glycol (0.1 mL). Controls received vehicle alone. This procedure was repeated twice more at 12-h intervals. Alternatively, 24 h after the last vitamin D_2 dose, the rats, under isoforane anesthesia (Ohio Medical Anesthetics, Madison, WI), were bilaterally nephrectomized or received sham operations. Immediately after these operations, they were dosed with propylene glycol (control) or vitamin D_3 metabolites (2 μg) exactly as described above. Six hours after the last dose, the rats in both groups were anesthetized with isoforane and killed by cardiac exsanguination. The plasma was collected in heparinized syringes and was analyzed for selected vitamin D_3 metabolites.

Plasma Metabolite Assay. 1,25-Dihydroxy[^{3}H]vitamin D_3, 1,23,25-$(OH)_3[^3H]D_3$, and 1,24,25-$(OH)_3[^3H]D_3$ (1000 cpm

each) were added to plasma (3–5 mL) to monitor recovery. The plasma lipids were extracted and applied to a Sephadex LH-20 column (0.6 × 15.5 cm) developed in water/chloroform/methanol/hexane (0.015:1:1:9). The 1,25-$(OH)_2D$ was eluted (12–20 mL) for HPLC purification and ultimate quantitation by competitive protein-binding analysis. The trihydroxy metabolites were eluted from the Sephadex LH-20 column by changing the solvent to water/chloroform/methanol/hexane (0.015:2:2:8) and were measured as described by Horst et al. (1983) by using the chick intestinal 1,25-$(OH)_2D$ receptor.

Prior to mass spectroscopy, the 1α-lactone, isolated from plasma of 1,23,25-$(OH)_3D_3$-treated rats, was reapplied to a Zorbax-NH_2 column developed in 2-propanol/hexane (14:86). The peak comigrating with 1α-lactone was recycled for a total of two passes across the column.

In Vitro Production of 1α-Lactone from (23S)-1,23,25-$(OH)_2D_3$. 1α-Lactone was produced from (23*S*)-1,23,25-$(OH)_3D_3$ by using chick kidney homogenates in exactly the same manner used for producing lactone from 25-OHD_3 (Horst, 1979). Briefly, the reactions were initiated by the addition of 0.06 μmol of (23*S*)-1,23,25-$(OH)_3D_3$ to each of 10 flasks. The mixture was purged with a stream of O_2 for 30 s, the flasks were sealed, and the mixture was incubated for 1.5 h at 37 °C with shaking. The reactions were quenched with 3.75 volumes of methanol/methylene chloride (2:1), and the lipid was extracted. The solvent was removed from the lipid extracts, and the residue was eluted with ethyl acetate through a column (0.6 × 10 cm) containing a mixture (1:1, w/w) of heat-activated silica acid (Bio-Rad, Richmond, CA) and Celite 503 (Baker, Phillipsburg, NJ). Twelve milliliters of eluent was collected and dried under N_2. The residue was dissolved in 300 μL of column solvent [2-propanol/hexane (17:83)] and was chromatographed on a normal-phase semipreparative column (0.9 × 25 cm) at a flow rate of 5 mL/min. The solvent containing the 1α-lactone fraction (55–65 mL) was dried under N_2, and the residue was applied to a normal-phase analytical column (0.45 × 25 cm) developed in 2-propanol/methylene chloride (7:93). The 1α-lactone fractions (16–18 mL) were collected; the solvent was evaporated; the residue was applied to a normal-phase analytical column developed in 2-propanol/hexane (14:86). The region corresponding to 1α-lactone (16–20 mL) was recycled twice for a total of three passes. The peak was collected for UV analysis and mass spectroscopy.

Plasma Pharmacokinetics. Male rats dosed with vitamin D_2 as above were each injected intravenously with 500 000 dpm (2.5 pmol) of either 1,23,25-$(OH)_3$[26,27-^{3}H]D_3 (90 Ci/mmol) (nine rats) or 1,25-$(OH)_2$[26,27-^{2}H]D_3 (nine rats). The weight range of the rats was from 320 to 340 g. The injections were prepared by dissolving ^{3}H metabolite in ethanol (50 μL) and suspending the dissolved materials in 3 mL of plasma taken from uninjected rats. Each rat received 0.3 mL of this mixture.

One milliliter of blood was taken from each of three rats for each time point. Blood was collected from the jugular vein under isoforane anesthesia with a heparinized syringe. The rats were divided into three groups, and bleeding times were staggered such that no rat was bled more than 4 times during the course of the experiment. The kinetic data were analyzed by Dr. Charles Ramberg at the University of Pennsylvania using the CONSAM (Boston et al., 1981), a conversational version of the SAAM computer program.

Silylation. 1,23,25-Trihydroxyvitamin D_3 (500 ng) was allowed to react with *N*-methyl-*N*-(trimethylsilyl)trifluoroacetamide (50 μL) at 90 °C for 2 h to affect persilylation. The silylated product was eluted at 15 mL with 0.1% dichloromethane in methanol from the HPLC column (Whatman ODS-3; 0.42 × 25 cm).

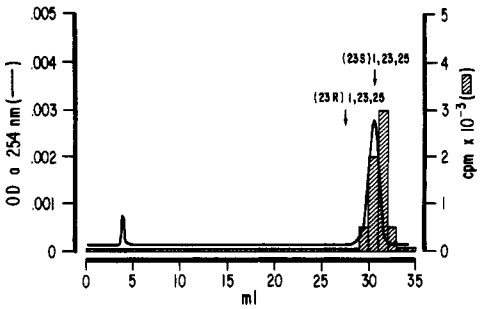

FIGURE 1: Elution of (23*S*)-1,23,25-$(OH)_3D_3$ and 1,23,25-$(OH)_3$-[^{3}H]D_3 generated in calf kidney homogenates from 1,25-$(OH)_2D_3$ and 1,25-$(OH)_2$[^{3}H]D_3 from a Zorbax NH_2 column developed in 2:8 2-propanol/hexane.

Biologic Evaluation of (23S)-1,23,25-$(OH)_3D_3$. Weanling male rats were housed individually in overhanging wire cages. They were fed vitamin D deficient diets containing low calcium (0.005%) and normal phosphorus (0.3%). After 3 weeks, the rats were anesthetized with ketamine, and the test compounds were given intrajugularly in 50 μL of a carrier solution containing ethanol/propylene glycol (3:7). Controls received the carrier solution alone. Twelve hours after injection, the rats were decapitated, and their duodena were used to measure intestinal calcium transport by the everted intestinal sac procedure (Martin & DeLuca, 1969). In addition, blood was collected and centrifuged. The resulting plasma was measured for calcium concentration by atomic absorption spectroscopy to determine the degree of bone calcium resorption. Since the rats were on a diet essentially devoid of calcium, serum calcium increases reflect mobilization of calcium from bone, not intestinal calcium absorption.

Statistics. The presence of significant differences was established by using Student's *t* test.

Results

Kidney homogenates of 1,25-$(OH)_2D_3$-dosed calves incubated with radioinert 1,25-$(OH)_2D_3$ produced a compound that comigrated on HPLC with chemically synthesized (23*S*)-1,23,25-$(OH)_3D_3$ (Figure 1). The purity of the radioinert 1,23,25-$(OH)_3D_3$ was denoted by the absence of peaks on the HPLC chromatogram that absorbed at 254 nm. Similarly, a radioactive compound that comigrated with radioinert (23*S*)-1,23,25-$(OH)_3D_3$ was produced when 1,25-$(OH)_2$-[^{3}H]D_3 was used as substrate (Figure 1). Again, purity was assessed by the absence of extraneous radioactive peaks in the 1,23,25-$(OH)_3$[^{3}H]D_3 region on the final HPLC column. The ultraviolet absorbance spectrum of the radioinert (23*S*)-1,23,25-$(OH)_3D_3$ displayed a maximum at 264 nm and a minimum at 229 nm. The ratio $\lambda_{max}/\lambda_{min}$ was 1.7 (data not shown). This is consistent with a pure metabolite containing a vitamin D-like cis-triene chromophore.

The EI mass spectrum of purified (23*S*)-1,23,25-$(OH)_3D_3$ (Figure 2) had a molecular ion at m/z 432, consistent with the addition of a hydroxy group to 1,25-$(OH)_2D_3$. Peaks at m/z 414, 396, and 378 represent sequential losses of water molecules from the molecular ion. The peak at m/z 381 represents loss of a methyl group from m/z 396. The peak at m/z 358 resulted from C-23/C-24 bond cleavage and is indicative of C-23 functionalized metabolites. Sequential losses of water molecules from m/z 358 produce m/z 340, 322, and 307. This side-chain fragmentation pattern was similar to that

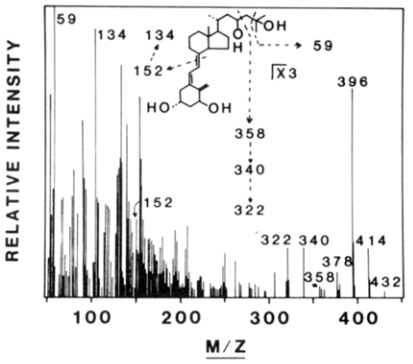

FIGURE 2: EI mass spectrum of (23*S*)-1,23,25-$(OH)_3D_3$ generated in calf kidney homogenates incubated with 1,25-$(OH)_2D_3$.

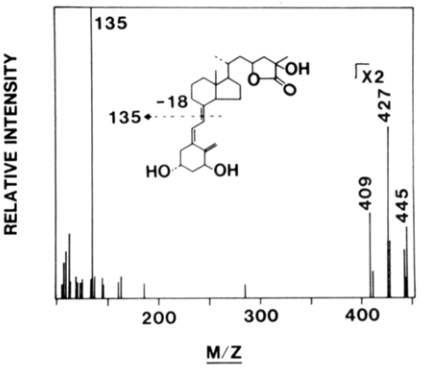

FIGURE 3: Cl-negative ion mass spectrum of 1α-lactone generated in chick kidney homogenates from (23*S*)-1,23,25-$(OH)_3D_3$.

of (23*S*)-23,25-$(OH)_2D_3$. The peak at m/z 152 (A ring plus C-6 and C-7) and the large peak at m/z 134 (m/z 152 minus water) are consistent with a 1α-hydroxylated vitamin D_3 metabolite. The base peak at m/z 59 was the result of C-24/C-25 bond cleavage. The peaks at m/z 155 and 105 are impurities that were also present in the standard, probably resulting from solvent contamination.

An EI spectrum of persilylated (23*S*)-1,23,25-$(OH)_3D_3$ had a peak at m/z 630 (1%) resulting from loss of $(CH_3)_3SiOH$ from the molecular ion. A peak at 540 (1.5%) represented loss of a $(CH_3)_3SiOH$ from the peak at m/z 630. A peak at m/z 206 (40%) was the silylated counterpart of m/z 134 (see above). The base peak at m/z 131 (100%) resulted from C-24/C-25 bond cleavage. These data reinforce the structural assignment as 1,23,25-$(OH)_3D_3$. The molecular weight of the metabolite was confirmed as 432 by Cl-NCI mass spectroscopy, which showed peaks at 467 (432 + ^{35}Cl, 100%) and 469 (432 + ^{37}Cl, 35%).

Having established that (23*S*)-1,23,25-$(OH)_3D_3$ is a 1,25-$(OH)_2D_3$ metabolite, we next investigated whether (23*S*)-1,23,25-$(OH)_3D_3$ and other vitamin D_3 metabolites were precursors to 1α-lactone. The results (Table I) clearly demonstrated that dosing rats with either 1,25-$(OH)_2D_3$ or (23*S*)-1,23,25-$(OH)_3D_3$, but not with (25*S*)-1,25,26-$(OH)_3D_3$, resulted in a significant elevation in plasma 1α-lactone concentrations. Also, on the basis of the molar amount of material injected, (23*S*)-1,23,25-$(OH)_3D_3$, of the metabolites tested, was clearly the most efficient precursor to 1α-lactone. The data also demonstrated that nephrectomy did not prevent the formation of 1α-lactone from 1,25-$(OH)_2D_3$ but rather resulted in higher concentrations relative to the sham-operated animals.

Other 1α-hydroxylated vitamin D_3 metabolites were also measured in the experimental animals. The results (Table I) demonstrated that plasma 1,24,25-$(OH)_3D_3$, 1,25,26-$(OH)_3D_3$, 1,23,25-$(OH)_3D_3$, and 1α-lactone were below the limits of detection in animals receiving vehicle alone (controls). Injection with 1,25-$(OH)_2D_3$ provided a substantial elevation of 1,25-$(OH)_2D_3$ in the plasma as well as an elevation in plasma 1,24,25-$(OH)_3D_3$ and 1,25,26-$(OH)_3D_3$. All of the detectable metabolites were higher in the nephrectomized animals when compared to their sham-operated controls. Plasma 1,23,25-$(OH)_3D_3$ concentrations were undetectable in all groups of animals except those dosed with (23*S*)-1,23,25-$(OH)_3D_3$. Nephrectomized animals dosed with (23*S*)-1,23,25-$(OH)_3D_3$ had higher concentrations of (23*S*)-1,23,25-$(OH)_3D_3$ than the sham-operated controls.

The procedure used to assay 1α-lactone in plasma employs a Sephadex LH-20 column and two HPLC columns to separate 1α-lactone from other vitamin D_3 metabolites. Nevertheless, in this work the 1α-lactone from plasma was isolated

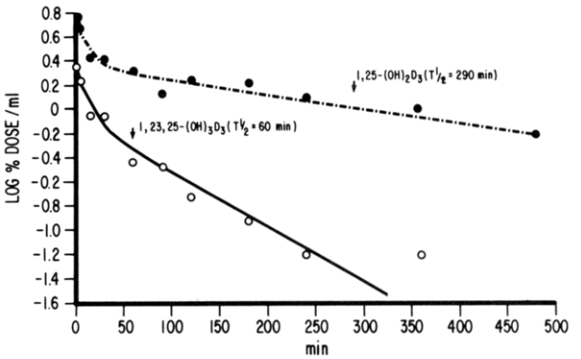

FIGURE 4: Plasma turnover of 1,23,25-$(OH)_3[^3H]D_3$ and 1,25-$(OH)_2[^3H]D_3$ in rats.

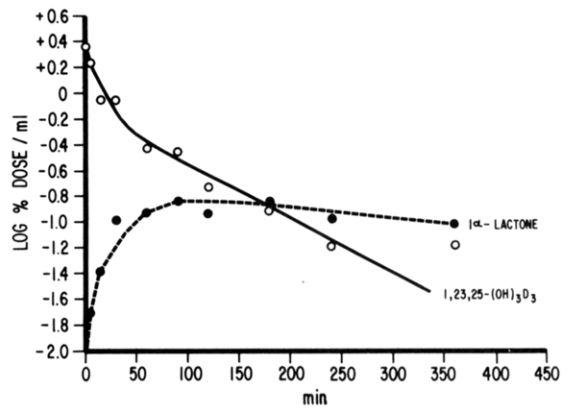

FIGURE 5: Time-dependent appearance of ^{3}H-1α-lactone following injection of 1,23,25-$(OH)_3[^3H]D_3$ to rats.

to confirm its identity. Material remaining after HPLC analysis was subjected to an additional HPLC step. The 1α-lactone recovered was analyzed by Cl-NCI mass spectroscopy. The molecular weight was confirmed by peaks at 479 (444 + ^{35}Cl, 100%) and 481 (444 + ^{37}Cl, 35%). 1α-Lactone isolated from chick kidney homogenates was further analyzed by PCI mass spectoscopy (Figure 3). It had a parent ion at 445 (444 + H^+), and sequentially showed loss of two molecules of water to give m/z 427 and 409. The base peak at m/z 135 (134 + H^+) was as expected for a 1α-hydroxylated vitamin D_3 metabolite. The molecular weight was confirmed as 444 by Cl-NCI mass spectroscopy which showed peaks at m/z 479 (444 + ^{35}Cl, 100%) and 481 (444 + ^{37}Cl, 35%).

The low plasma (23*S*)-1,23,25-$(OH)_3D_3$ relative to the plasma 1,25-$(OH)_2D_3$ prompted experiments to determine the plasma turnover of these metabolites. The results (Figure 4) demonstrated that a tracer dose (approximately 1 ng) of 1,23,25-$(OH)_3[^3H]D_3$ was clearly from plasma 5 times faster

Table I: Vitamin D Metabolite Concentrations in the Plasma in Vitamin D_2 Treated Rats Dosed with Vitamin D_3 Metabolites [Each Value Representing the Mean ± SD (n = 4)]

		pg/mL			ng/mL	
compound dosed[a]	operation[b]	1,25-$(OH)_2D_3$	1,24,25-$(OH)_3D_3$	1,25,26-$(OH)_3D_3$	1,23,25-$(OH)_3D_3$	1α-lactone
control	sham	90 ± 17	<10	<10	<0.15	<1.0
	Nx[c]	284 ± 97	<10	<10	<0.15	<1.0
1,25-$(OH)_2D_3$	sham	4900 ± 800	46 ± 13	15 ± 4	<0.15	9.4 ± 12
	Nx	26200 ± 8800[d]	205 ± 50[d]	59 ± 10[d]	<0.15	15.0 ± 1.6
1,23,25-$(OH)_3D_3$	sham	29 ± 4	<10	<10	0.25 ± 0.03	33 ± 1.5
	Nx	22 ± 2	<10	<10	1.23 ± 0.6[d]	105 ± 53[d]
1,25,26-$(OH)_3D_3$	sham	80 ± 4	<10	270 ± 20	<0.15	<1.0

[a] Each animal received a total of three 2-μg doses of the appropriate vitamin D_3 metabolites. [b] Refer to section on 1α-lactone precursors for details. [c] Nx = nephrectomy. [d] Within treatment group significantly different from sham, $p < 0.05$.

Table II: Bone Calcium Mobilization and Intestinal Calcium Transport in Rats Administered (23S)-1,23,25-Trihydroxyvitamin D_3 and 1,25-Dihydroxyvitamin D_3[a]

dose	^{45}Ca serosal/ ^{45}Ca mucosal	plasma calcium
Experiment I		
control	2.9 ± 0.7	4.3 ± 0.2
1,25-dihydroxyvitamin D_3		
6.25 ng	4.5 ± 0.4[b]	4.5 ± 0.4
12.5 ng	5.0 ± 1.2[b]	4.9 ± 0.3[b]
(23S)-1,23,25-trihydroxyvitamin D_3		
50 ng	2.9 ± 0.8	4.4 ± 0.3
100 ng	3.0 ± 0.8	4.4 ± 0.1
200 ng	3.0 ± 0.8	4.2 ± 0.6
500 ng	3.3 ± 0.8	4.0 ± 0.3
Experiment II		
control	2.3 ± 0.3	4.6 ± 0.2
1,25-dihydroxyvitamin D_3, 12.5 ng	4.2 ± 0.8[b]	4.9 ± 0.2[b]
1,25-dihydroxyvitamin D_3 (12.5 ng) + (23S)-1,23,25-trihydroxyvitamin D_3 (100 ng)	4.1 ± 0.8[b]	5.0 ± 0.2[b]

[a] n = 5 for each treatment group. [b] Significantly different from control, $p < 0.05$.

than the same dose of 1,25-$(OH)_2[^3H]D_3$. Concurrent with the disappearance of 1,23,25-$(OH)_3[^3H]D_3$ was the appearance of ^{3}H-1α-lactone (Figure 5). Pharmacokinetic estimates suggest that 10% of the 1,23,25-$(OH)_3[^3H]D_3$ disappeared in the process of ^{3}H-1α-lactone formation. *The pharmacokinetic model, therefore, suggests that approximately 90% of plasma 1,23,25-$(OH)_3[^3H]D_3$ appeared to be removed from plasma by pathways other than via the formation of ^{3}H-1α-lactone.*

Biological Evaluation. The intestinal calcium transport responses of rats that received a single dose of test compound 12 h prior to experiment are recorded in Table II. The data suggest that at levels of up to 500 ng, (23S)-1,23,25-$(OH)_3D_3$ has no intestinal calcium absorptive or bone calcium resorptive activity. The compound 1,25-$(OH)_2D_3$ had significant activity at the 6.25-ng dose. In addition, no significant anti-vitamin D activity was observed when 1,25-$(OH)_2D_3$ (12.5 ng) was codosed with 1,23,25-$(OH)_3D_3$ (100 ng).

The data in Figure 6 demonstrate the affinity of (23S)-1,23,25-$(OH)_3D_3$ in the chick intestinal cytosol receptor assay. (23S)-1,23,25-Trihydroxyvitamin D_3 was 60-fold less competitive than 1,25-$(OH)_2D_3$.

Discussion

This report demonstrates that (23S)-1,23,25-$(OH)_3D_3$ is a significant 1,25-$(OH)_2D_3$ metabolite in kidney of homogenates prepared from 1,25-$(OH)_2D_3$-dosed calves (Figure 7). The structural assignment as (23S)-1,23,25-$(OH)_3D_3$ was determined by chromatographic and spectral means and by comparing the natural product with synthetic standards. The new

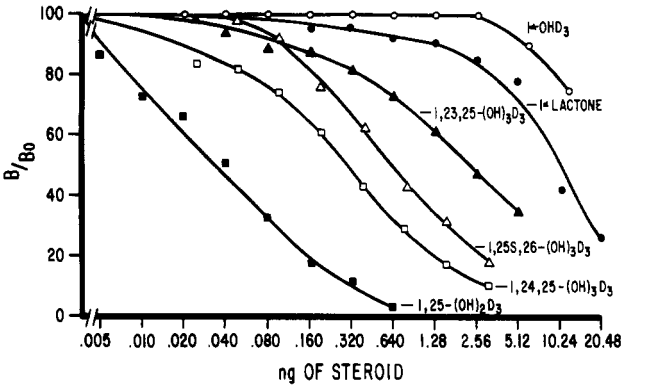

FIGURE 6: Relative binding affinities for 1α-hydroxylated vitamin D metabolites using the chick intestinal cytosol receptor.

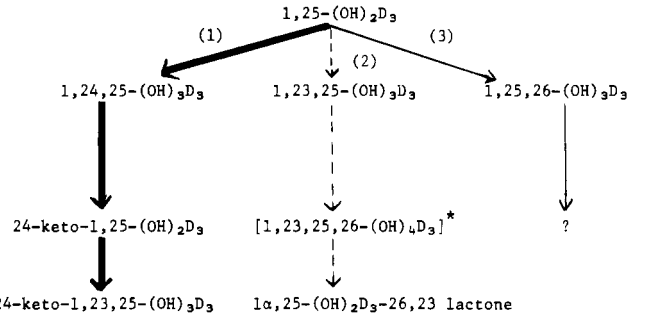

FIGURE 7: Pathways for the metabolism of 1,25-$(OH)_2D_3$. (Asterisk) Postulated intermediate based on Napoli & Horst (1983a). (1) Heavy solid line is the major physiologic pathway involving the kidney and intestine. (2) Dashed line is the major pharmacologic pathway, which *does not* involve kidney and intestine, and a minor physiologic pathway in kidney and intestine. (3) Light solid line is a minor physiologic and pharmacologic pathway; major organs involved in synthesis are unknown.

metabolite was shown to have a molecular weight of 432 by electron impact and chloride addition, negative ion chemical ionization mass spectroscopy. An ultraviolet absorbance spectrum indicated that the metabolite contained a *cis*-vitamin D 5,7,10(19)-triene chromophore. This was confirmed by the fragmentation pattern observed in the electron impact mass spectrum, which also indicated that the compound was a side-chain hydroxylated 1,25-$(OH)_2D_3$ derivative. Finally, a mass spectrum of the silylated derivative confirmed the presence of four hydroxyl groups and provided further evidence that the side-chain hydroxyl groups were on C-23 and C-25.

The results of in vivo experiments showed that (23S)-1,23,25-$(OH)_3D_3$, but not (25S)-1,25,26-$(OH)_3D_3$, is a precursor to 1α-lactone. Therefore, 23S-hydroxylation of 1,25-$(OH)_2D_3$ appears to be the initial step in the formation of 1α-lactone from 1,25-$(OH)_2D_3$. Initial hydroxylations and subsequent formation of 1α-lactone from 1,25-$(OH)_2D_3$, therefore, parallel the events required for the formation of

lactone from 25-OHD_3 (Napoli & Horst, 1981; Napoli et al., 1982). Nephrectomy has been shown to abolish the formation of lactone in acutely vitamin D_3 toxic pigs and greatly reduce the formation of lactone in rats dosed with 25-OHD_3 (Horst & Littledike, 1980; Napoli et al., 1982). In the present experiment, nephrectomized rats dosed with 1,25-$(OH)_2D_3$ had greater concentrations of 1α-lactone than the similarly treated sham-operated control. Nephrectomy, therefore, did not compromise the ability of the rats to form 1α-lactone but did increase the plasma $t_{1/2}$ of 1,25-$(OH)_2D_3$. Comparison of plasma 1,25-$(OH)_2D_3$ in nephrectomized animals with that in sham-operated controls demonstrated at least 5 times more circulating 1,25-$(OH)_2D_3$ in the former group. The higher 1,25-$(OH)_2D_3$ concentrations could be responsible for the higher plasma 1α-lactone as well as 1,24,25-$(OH)_3D_3$ and 1,25,26-$(OH)_3D_3$. These data, therefore, provide evidence that pharmacologically C-23, C-24, and C-26 oxidation of 1,25-$(OH)_2D_3$ occur extrarenally. C-24 hydroxylation of 25-OHD_3 and 1,25-$(OH)_2D_3$ has been shown to take place in cartilage (Garabedian et al., 1978) and intestine (Kumar et al., 1978; Napoli et al., 1983). C-23 and C-26 oxidation of 1,25-$(OH)_2D$ does occur in the intestine at physiologic substrate concentrations (Napoli & Horst, 1983a,b); however, the organ site of the quantitatively most significant production is not yet clear.

The kinetic data suggest that, when formed, the 1,23,25-$(OH)_3[^3H]D_3$ is rapidly cleared from plasma and that formation of ^{3}H-1α-lactone from 1,23,25-$(OH)_3[^3H]D_3$ is responsible for only a minor (10%) part of the plasma clearance of 1,23,25-$(OH)_3D_3$. The rapid plasma clearance of 1,23,25-$(OH)_3D_3$, in addition to its lack of biologic activity and receptor binding, suggests that C-23 hydroxylation may be an initial step in the vivo deactivation and metabolic clearance of excess 1,25-$(OH)_2D_3$.

Presently, there is little information concerning the primary organs involved in C-23 oxidation. The present experiments with rats showed that 1α-lactone, like lactone, could clearly be synthesized in the absence of kidneys. Napoli et al. (1983), Napoli & Horst (1983a,b), and Napoli & Martin (1984) have shown that expression of C-23 hydroxylase activity in both kidney and intestine apparently occurs with mainly C-24-oxidized 1,25-$(OH)_2D_3$ metabolites as substrates; i.e., C-23 oxidation of 1,25-$(OH)_2D_3$ appears to be a quantitatively minor pathway under physiologic conditions. Production of 1α-lactone was not enhanced in cell cultures of these organs when high substrate concentrations were used. The majority of 1α-lactone production, therefore, likely represents a nonrenal, nonenteric oxidative pathway, the expression of which is augmented greatly by but not limited to states of 1,25-$(OH)_2D_3$ excess. On the other hand, these data do not necessarily eliminate C-23 oxidation of 1,25-$(OH)_2D_3$ as a major primary physiologic pathway of 1,25-$(OH)_2D_3$ metabolism, particularly since it is rapidly metabolized. As shown by our kinetic data, 1,23,25-$(OH)_3D_3$ is cleared rapidly from plasma to give rise to only a small amount of 1α-lactone, which is cleared approximately 10 times slower. It is also clear from our data that significant amounts of 1α-lactone can appear in plasma under circumstances where its precursor, (23*S*)-1,23,25-$(OH)_3D_3$, cannot be detected. Therefore, in physiologic states C-23 oxidation could be a significant primary pathway of 1,25-$(OH)_2D_3$ metabolism; however, its manifestation would be precluded by the rapid removal of 1,23,25-$(OH)_3D_3$ from plasma to either 23 acid or unknown metabolites.

In summary, we have identified (23*S*)-1,23,25-$(OH)_3D_3$ as a renal and extrarenal metabolite of 1,25-$(OH)_2D_3$ which is cleared rapidly from plasma and is a precursor to 1α-lactone. The physiologic significance of the C-23 oxidation of 1,25-$(OH)_2D_3$ is under investigation in our laboratories. Our current hypothesis, supported by the data presented here, is that C-23 oxidation is a significant route of 1,25-$(OH)_2D_3$ inactivation and plays an important role in maintaining 1,25-$(OH)_2D_3$ homeostasis.

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An Antibody Probe To Determine the Native Species of Glycinamide Ribonucleotide Transformylase in Chicken Liver†

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ABSTRACT: Antibody probes of Western blots [Renart, J., Reiser, J., & Stark, G. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 3116] of chicken liver homogenates under various conditions revealed that glycinamide ribonucleotide transformylase can be rapidly proteolyzed in such homogenates. These findings, along with molecular weight measurements by ultracentrifugation, identify the true form of glycinamide ribonucleotide transformylase as a monomeric protein of 117 000 daltons. This protein has been purified 400-fold in 44% yield from chicken liver in one step on an affinity column of 10-formyl-5,8-dideazafolate–Sepharose. Native glycinamide ribonucleotide transformylase retains full activity after proteolytic cleavage to a form (M_r 55 000) similar to fragments seen in the Western blot of the homogenates. This phenomenon may be responsible for the previous identification of glycinamide ribonucleotide (GAR) transformylase as a dimer of 55 000-dalton subunits. Similar analyses using antibodies to 5-aminoimidazole-4-carboxamide ribonucleotide (AICAR) transformylase [Mueller, W. T., & Benkovic, S. J. (1981) *Biochemistry 20*, 337] and trifunctional enzyme [Smith, G. K., Mueller, W. T., Wasserman, G. F., Taylor, W. D., & Benkovic, S. J. (1980) *Biochemistry 19*, 4313] confirm that these two proteins were isolated in their native forms.

Glycinamide ribonucleotide transformylase (GAR transformylase, EC 2.1.2.2)[1] catalyzes the transfer of a formyl group from (6*R*)-10-CHO-H_4folate to glycinamide ribonucleotide and is one of two such reduced-folate-requiring enzymes in the purine biosynthetic pathway. This enzyme has been purified previously by Warren & Buchanan (1957) and subsequently by workers in this laboratory (Caperelli et al., 1978, 1980). The latter group associated the transformylase activity with a homodimer protein of 55 000-dalton subunits on the basis of the results of sucrose density ultracentrifugation and SDS–polyacrylamide gel electrophoresis studies. Smith et al. (1980) have shown that GAR transformylase activity copurifies through several steps with another enzyme: the 5,10-methylene-, 5,10-methenyl-, and 10-formyl-H_4folate synthetase (combined) (trifunctional enzyme, EC 6.3.4.3, EC 1.5.1.5, and EC 3.5.4.9), which catalyzes interconversions between reduced one-carbon-substituted folate cofactors.

In this paper we report that GAR transformylase is a single-subunit protein of about 117 000 daltons in chicken liver. Evidence in support of this conclusion comes primarily from Western blot analysis of trichloroacetic acid homogenized liver extracts. Probes of the same blots with antibodies to AICAR transformylase (EC 2.1.2.3; Mueller & Benkovic, 1981) and trifunctional enzyme indicate that the native subunit molecular weight of these proteins is 67 000 and 95 000, respectively. These subunit molecular weights are in good agreement with those obtained previously. The 117 000-dalton protein has been purified to near homogeneity in a single chromatographic step on a column of 10-formyl-5,8-dideazafolate–Sepharose, which has been employed previously in the purification of thymidylate synthetase from L1210 mouse leukemia cells by Rode et al. (1979). We present evidence for a limited in vitro proteolysis to the 55 000-dalton form that retains full activity, indicating that this cleavage may have been responsible for the previous identification of GAR transformylase as a dimer.

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[1] Abbreviations: GAR, α,β-glycinamide ribonucleotide; H_4folate, tetrahydrofolate; 5,10-C^+H-H_4folate, (6*R*)-5,10-methenyltetrahydrofolate; 10-CHO-H_4folate, (6*R*)-10-formyltetrahydrofolate; 2-ME, 2-mercaptoethanol; GAR transformylase, (6*R*)-10-formyltetrahydrofolate:5′-phosphoribosylglycinamide formyltransferase; trifunctional enzyme, 5,10-methenyltetrahydrofolate, 5,10-methylenetetrahydrofolate, and 10-formyltetrahydrofolate synthetase (combined); SDS, sodium dodecyl sulfate; NP-40, Nonidet P-40; FGAR, N^β-formylglycinamide ribonucleotide; AICAR, 5-aminoimidazole-4-carboxamide 1-ribonucleotide; AICAR transformylase, (6*R*)-10-formyltetrahydrofolate:5′-phosphoribosyl-5-amino-4-imidazolecarboxamide formyltransferase; anti-GAR, antiserum to GAR transformylase; anti-AICAR, antiserum to AICAR transformylase; anti-TP, antiserum to trifunctional protein; TCA, trichloroacetic acid; APT, (aminothio)phenol; DTT, dithiothreitol; EDTA, ethylenediaminetetraacetic acid; Tris, tris(hydroxymethyl)-aminomethane.

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